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Antisense Oligodeoxynucleotides to the Kappa$_1$ Receptor Enhance Δ^9-THC–Induced Antinociceptive Tolerance

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ROWEN, D. W., J. P. EMBREY, C. H. MOORE AND S. P. WELCH. *Antisense oligodeoxynucleotides to the kappa$_1$ receptor enhance Δ^9-THC–induced antinociceptive tolerance*. PHARMACOL BIOCHEM BEHAV **59**(2) 399–404, 1998.—Delta-9-tetrahydrocannabinol produces potent antinociceptive effects in mice and rats. Evidence exists for an interaction between the cannabinoids and the kappa receptor subtype, kappa$_1$, in the production of antinociception. Data indicate that Δ^9-THC induces the release of endogenous dynorphins, the ligand(s) for the kappa receptor. It has been demonstrated that antisense oligodeoxynucleotides directed against the kappa$_1$ receptor attenuate the antinociceptive effects of Δ^9-THC. The exact mechanism for the expression of cannabinoid tolerance is unknown. Bidirectional cross-tolerance between the kappa opioids and Δ^9-THC implies that a common mechanism may be responsible for tolerance expression. We tested the hypothesis that the kappa$_1$ receptor is involved in tolerance to Δ^9-THC. Antisense to the kappa$_1$ receptor has been shown to downregulate the kappa receptor. We observed a significant increase in the ED_{50} for Δ^9-THC in antisense-, but not mismatch-treated mice, indicating an increase in tolerance to Δ^9-THC. Such data indicate that a decrease in kappa receptor number may accompany tolerance to Δ^9-THC. © 1998 Elsevier Science Inc.

Kappa Δ^9-THC Antisense Dynorphin Receptors Tolerance Antinociception Spinal

THE major psychoactive ingredient in marijuana is the cannabinoid delta-9-tetrahydrocannabinol (Δ^9-THC). Delta-9-tetrahydrocannabinol and other cannabimimetic drugs produce a variety of pharmacological effects on the central nervous system that include antinociception, hypoactivity, catalepsy, and hypothermia (10). Cannabinoid receptors have been cloned from brain (18) and macrophages from the spleen (20). The putative endogenous ligand for these receptors has been identified: arachidonylethanolamide (anandamide), an arachidonic acid derivative (9). The antinociceptive effects of Δ^9-THC can be pharmacologically separated from other behavioral effects (29). Such results imply that a separate mechanism exists for Δ^9-THC–induced antinociception, relative to the other behavioral effects of the drug, thus possibly allowing for the separation of undesirable side effects in the treatment of pain.

Delta-9-tetrahydrocannabinol produces potent antinociceptive effects in mice and rats through both spinal and supraspinal mechanisms (16,28,37,39). There is a high-affinity binding of the cannabinoids in the substantia gelantinosa of the spinal cord (13), an area associated with dense binding of the opiates (42). The opioids and the cannabinoids share many of the same pharmacological properties such as analgesia, sedation, and euphoria (10). Evidence exists for a link between the cannabinoids and the opioid system (30,40) via an interaction between the cannabinoids and the kappa receptor subtype, kappa$_1$ in the production of antinociception. Δ^9-THC enhances the antinociceptive effects of the opioids administered intrathecally (IT) (39), and bidirectional cross-tolerance between Δ^9-THC and kappa agonists has been demonstrated (29). Welch (37) has shown that the antinociception produced by IT administration of Δ^9-THC is blocked by the kappa receptor antagonist, norbinaltorphimine (nor-BNI) and the kappa$_1$ receptor antagonist, naloxone benzoylhydrazone (Nal-BZH) (38). However, binding studies using the mouse spinal cord and brain indicate that a direct interaction of the kappa

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antagonist, nor-BNI, at the cannabinoid receptor does not occur because nor-BNI and NalBZH fail to displace cannabinoid (^{3}H-CP55,940) binding at the cannabinoid receptor (37). Mu and delta receptor antagonists fail to block Δ^9-THC–induced antinociception, also supporting a kappa receptor interaction (37). Pugh et al. (23) provide evidence for an indirect interaction between the kappa receptor and Δ^9-THC in a study in which the antinociception effects of Δ^9-THC are attenuated with antisera to Dynorphin A (1–8) and Dynorphin A (1–17). Furthermore, the data indicate that Δ^9-THC induces the release of endogenous dynorphins, the ligand(s) for the kappa receptor. High levels of dynorphins are found in the dorsal horn of the spinal cord. This indicates that spinal dynorphins or leucine enkephalin, a metabolite of dynorphin, have a role in Δ^9-THC induced spinal antinociception.

Antisense strategies are proving to be a valuable pharmacological tool. The mu, delta, and kappa$_1$ opioid receptor subtypes have all been cloned (6,19,43,45). Antisense oligodeoxynucleotides targeted against these opioid receptors, as well as the associated G proteins, have demonstrated the utility of antisense strategies in the study of the nociceptive pathway (6,24,27,30). It has been demonstrated that antisense oligodeoxynucleotides directed against the kappa$_1$ receptor block the antinociceptive effects of Δ^9-THC, substantiating the hypothesis that Δ^9-THC–induced spinal antinociception is due in part to the release of spinal dynorphins (22).

Chronic exposure to Δ^9-THC results in the expression of tolerance to Δ^9-THC–induced antinociception, hypoactivity, and hypothermia in mice (11,29). The exact mechanism for the expression of cannabinoid tolerance is unknown. The bidirectional cross-tolerance noted between the kappa opioids and Δ^9-THC implies that a common mechanism may underlie tolerance to both classes of drugs (29,34). In addition, cannabinoid-induced downregulation of the cannabinoid receptor has been shown. Oviedo et al. (21) found that following the chronic administration of Δ^9-THC and the cannabinoid, CP55,940, in rat brains, the B_{max} was reduced by 50 to 60%. Corresponding to the reduction in receptor number, a comparable increase in tolerance was noted. Rodriguez De Fonseca et al. (26) also reported downregulation of cannabinoid binding sites in the rat brain following chronic exposure to Δ^9-THC. However, agonist-induced receptor downregulation is not always associated with the expression of tolerance. Abood et al. (1) found no changes in cannabinoid binding in the brains of tolerant vs. nontolerant mice. Westlake et al. (41) demonstrated that chronic administration of Δ^9-THC failed to produce an irreversible alteration in cannabinoid receptors in the rat brain. These studies support the theory that tolerance to cannabinoids may involve mechanisms other than receptor cannabinoid receptor downregulation.

Utilizing antisense oligodeoxynucleotides to the kappa$_1$ receptor subtype, it was hypothesized that the expression of tolerance to Δ^9-THC could be enhanced in mice. Our hypothesis is that the kappa$_1$ receptor is involved in the pathway responsible for the expression of tolerance to Δ^9-THC. Antisense to the kappa$_1$ receptor would presumably decrease kappa receptor numbers, thus eliminating a portion of the kappa receptors from the biochemical pathway (dynorphin release and binding to the kappa receptor) speculated to be responsible for the production of antinociception by Δ^9-THC.

METHOD

Male ICR mice (Harlan Laboratories, Indianapolis, IN), which weighed 20 to 31 g, were used. The mice were maintained on a 12 L: 12 D cycle with free access to food and water, five mice per cage. The mice (n = 240) were equally divided into two groups; the first receiving chronic Δ^9-THC injections subcutaneous (SC); and the second as a control. Drugs were obtained from the sources indicated: Δ^9-THC (National Institute on Drug Abuse, Rockville, MD), and kappa$_1$ receptor antisense and mismatch oligodeoxynucleotides (DNA International).

The mice were rendered tolerant to the effects of Δ^9-THC by repetitive administration of 10 mg/kg of Δ^9-THC over a 7-day period using the methods of Smith et al. (29). The Δ^9-THC was dissolved in 1:1:18 (emulphor:ethanol:saline) for SC injections. The control group received SC injections of the vehicle, 1:1:18 (emulphor:ethanol:saline). The animals received 13 injections Δ^9-THC over the period of 7 days. The injections were administered as follows: two SC injections per day with dosing starting at 0800 h and 1600 h for the first 6 days and a single injection with dosing commencing at 0800 h on day 7. All animals were injected within a 120-min period and in approximately same order each day. On day 8, Δ^9-THC–induced antinociception was assessed in the tail-flick test 24 to 26 h after the last injection of Δ^9-THC. The animals showed no weight loss with this treatment paradigm.

The two groups (Δ^9-THC and control) were further subdivided into three groups containing 40 mice each. The first group received an antisense oligodeoxynucleotide directed against the kappa$_1$ receptor (5′GGTGCCTCCAAGGACTATCGC-3′). The second group received the kappa$_1$ receptor mismatched oligodeoxynucleotide that contained four bases that were switched (5′GGAGCCTGCAAGGTCTATGGC-3′). The third group, the control, received distilled water. The kappa$_1$ receptor antisense and mismatch were diluted in distilled water to a concentration of 5 μg/5 μl. The injections were administered IT, on days 3, 5, and 7, commencing at 0800 h, according to the protocol of Chien et al. (7). The IT injections were performed according to the protocol of Hylden and Wilcox (14) and required approximately 3 h to complete for all groups. Unanesthetized mice were injected between the L5–L6 area of the spinal cord with a 30-gauge, ½ inch needle, attached to 50-μl Hamilton syringe. Injection volumes of 5 μl were administered.

Delta-9-tetrahydrocannabinol-induced antinociception was assessed on day 8 in all mice at 24 h after the last injection of Δ^9-THC or vehicle. Delta-9-tetrahydrocannabinol was dissolved in 100% dimethyl sulfoxide (DMSO) for IT injections. DMSO (100%) was used as the vehicle in the control groups. DMSO produced some scratching had hyperirritability in the mice for 2 min after the injection, although DMSO did not produce antinociceptive effects (<14%) MPE). Antinociception was assessed using the tail-flick latency test of D'Amour and Smith (8). The heat lamp of the tail-flick apparatus was maintained at an intensity sufficient to produce control latencies of 2–4 s. Control values for each animal were determined before Δ^9-THC (IT) administration. The mice were then retested 10 min after IT injections and latencies to the tail-flick responses were recorded. A 10-s maximum was imposed to prevent tissue damage. Antinociception was quantified as the percent maximum possible effect (% MPE) as developed by Harris and Pierson (12) using the formula:

$$\%\ \text{MPE} = \frac{[\text{Test latency} - \text{control latency}]}{[10 - \text{control latency}]} \times 100$$

Percentage of MPE was calculated for each mouse using at least six mice per dose. By using the % MPE for each mouse,

the mean effect and standard error mean (SEM) was calculated for each dose. Dose–response curves were generated using at least three doses of the test drug. ED_{50} values were determined by log-probit analysis, and 95% confidence limits (CLs) were determined using the method of Litchfield and Wilcoxon (17). A significant statistical difference was determined by a lack of overlap in the confidence limits generated for the ED_{50}s.

RESULTS

The antinociceptive effects of Δ^9-THC were evaluated in the tail-flick test as described above. The ED_{50}s for all six treatment groups (see Table 1) were obtained using a minimum of three doses for each dose–response curve (see Figs. 1 and 2). An ED_{50} (μg/mouse, ±95% confidence limits, CLs) of 61 [33–78] μg/mouse was obtained for the vehicle/distilled water group vs. 180 [114–203] for the Δ^9-THC/distilled water group. Thus, the mice exhibited threefold tolerance to Δ^9-THC. An ED_{50} of 61 [47–113] μg/mouse was obtained for the vehicle/kappa$_1$ mismatch group vs. 196 [103–228] of the Δ^9-THC/kappa$_1$ mismatch group. Thus, the mice exhibited threefold tolerance to Δ^9-THC as had been observed for vehicle-pretreated mice (Fig. 1). An ED_{50} of 114 [83–194] μg/mouse was observed in the vehicle/kappa$_1$ antisense group, which was significantly different from the chronic vehicle/vehicle (IT) pretreated mice (ED_{50} = 61 [33–78]). The 1.9-fold shift in ED_{50} for Δ^9-THC (from 61 to 113 μg/mouse) in the "chronic vehicle group" is consistent with the attenuation of the effects of Δ^9-THC by kappa antisense (22). An ED_{50} of 114 [83–184] μg/mouse was observed in the vehicle/kappa$_1$ antisense group versus 514 [401–623] μg for the Δ^9-THC/kappa$_1$ antisense group. The 4.5-fold shift in ED_{50} from 114 to 514 μg/mouse is a significant difference in that the 95% CLs of the ED_{50} do not overlap. The 2.9-fold ED_{50} shift from 180 to 514 μg/mouse also represents a significant change in ED_{50}s between the two groups.

It should be noted that in doses higher than 200 μg/mouse Δ^9-THC, approximately 20% of the mice were noted to exhibit hyperexcitability when stimulated, catalepsy, hypoactivity, and splaying of their hindquarters. As the dose increased, the incidence of this behavior was noted to increase to nearly 50% of the mice. However, the mice were able to flick their tails. Had the motor effects delayed the "flick" response, one would expect that high doses of Δ^9-THC would elicit increased levels of antinociception. We observed just the opposite effect, in that even higher doses of Δ^9-THC were needed in the Δ^9-THC–tolerant mice to elicit antinociception. Thus, we concluded that the motor impairment was not responsible for the high dose of Δ^9-THC required to elicit antinociception in the "antisense/Δ^9-THC–tolerant" group.

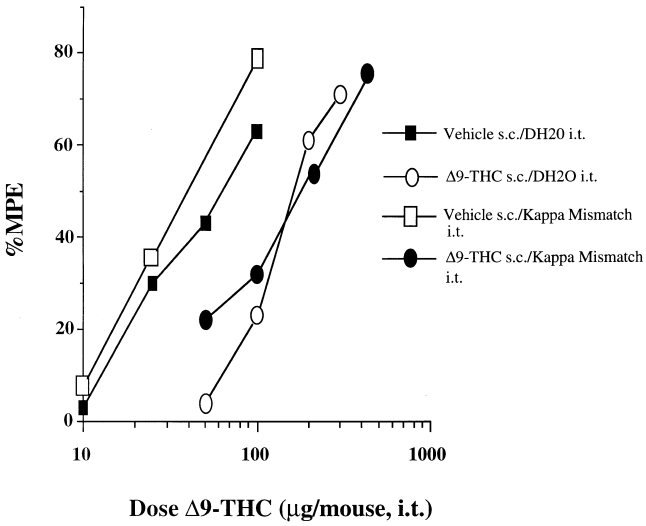

FIG. 1. Dose–response curves for Δ^9-THC following pretreatment with kappa$_1$ mismatch and controls. Antinociception was assessed using the tail-flick method as described under the Methods Section using at least an $n = 6$ mice per dose.

TABLE 1

ED50S FOR △9-THC (IT) IN PLACEBO AND △9-THC–TOLERANT MICE PRETREATED WITH EITHER KAPPA$_1$ ANTISENSE, MISMATCH, OR VEHICLE (IT, AS IN THE METHOD SECTION)

	ED_{50}s for △9-THC in μg/Mouse	
Cotreatment	Chronic vehicle (SC)	Chronic △9-THC (SC)
vehicle (distilled water, IT)	61 (33–78)	180 (114–203)
Mismatch IT	61 (47–113)	196 (103–228)
Antisense IT	114 (83–184)*	514 (401–623)*

Mice were pretreated with "chronic vehicle" which was 1:1:18 (emulphor:ethanol:saline, SC) or chronic △9-THC (SC) as described in the Method Section. Concurrently mice were injected IT with either vehicle (distilled water), mismatch oligonucleotide, or antisense oligonucleotide every 48 h for 6 days (a total of three injections). On day 8 of the protocol the ED_{50} for △9-THC (IT in DMSO vehicle) was determined in all of the mice. Separate groups of mice in all treatment groups were tested for antinociception following DMSO (IT) administration and the %MPE generated was uniformly less than 14%.

*Significantly different from vehicle group (no overlap of 95% confidence limits).

DISCUSSION

Our data demonstrate that kappa$_1$ antisense administration blocks the antinociceptive effects of Δ^9-THC administrated (IT), thus supporting previous findings (22). In addition, the following findings support our hypothesis linking dynorphin to spinal antinociception produced by the cannabinoids, and to the enhancement of opiate antinociception by the cannabinoids: 1) the antinociceptive effects of Δ^9-THC, but not hypothermia, catalepsy, or hypoactivity, are blocked by antisera to dynorphin A [1–8] and dynorphin A [1–17] (23). These results parallel those observed with the nor-BNI blockade of cannabinoid-induced antinociception, but not hypothermia, hypoactivity, or catalepsy (29). 2) The inhibition of dynorphin metabolism attenuates the enhancement of morphine-induced antinociception in the presence of the cannabinoids (23). Thus, the breakdown of dynorphin to leucine enkephalin may result in the greater-than-additive antinoci-

Figures 1 and 2 indicate the dose–response curves from which the ED_{50}s in Table 1 were derived. Figure 1 indicates the effect of the mismatch administration on the antinociceptive effects of Δ^9-THC. Figure 2 shows the effects of the antisense administration on the effects of Δ^9-THC. Dose–effect curves did not differ significantly from parallelism, as determined by the method of Tallarida and Murray (31).

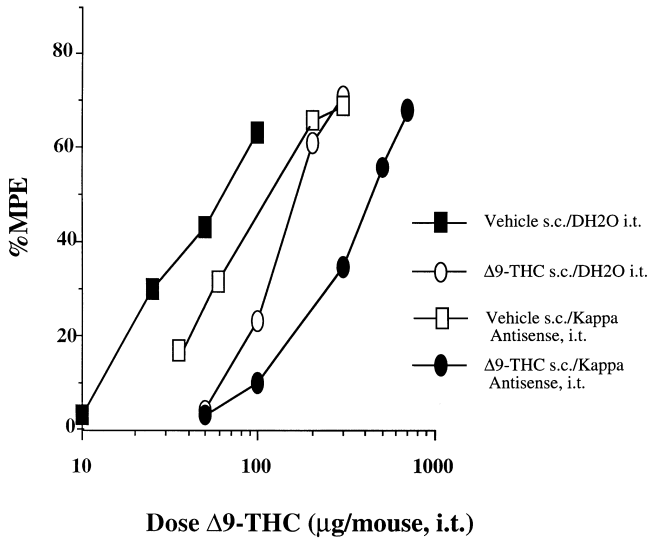

FIG. 2. Dose–response curves for Δ^9-THC following pretreatment with kappa$_1$ antisense and controls. Antinociception was assessed using the tail-flick method as described under the Methods Section using at least an $n = 6$ mice per dose.

ceptive effects of morphine and cannabinoids (IT). 3) The greater-than-additive antinociceptive effects of the combination of the cannabinoids and morphine (40) can be totally blocked by naltrindole, a delta opioid antagonist, at a dose that blocks neither morphine, nor Δ^9-THC, nor kappa agonists. Thus, the only point for the blockade by naltrindole would be at the delta receptor, indicating the production of a delta opioid peptide, such as leucine enkephalin. This supports the observation that the antinociceptive effects (IT) of the delta agonist, DPDPE ([D-Pen^2,D-Pen^5] enkephalin), are greatly enhanced by Δ^9-THC administration (40). The total block of the cannabinoid/morphine interaction by nor-BNI (29,37), which is hypothesized to be blocking the kappa opioid, dynorphin, clearly implicates the release of dynorphin by the cannabinoids in the production of antinociception and enhancement of morphine (35).

The function of the mismatch oligodeoxynucleotide is to verify the specificity of the base sequence of the antisense oligodeoxynucleotide for the mRNA under investigation (36). Therefore, both kappa$_1$ mismatch groups (vehicle and Δ^9-THC) theoretically should have ED$_{50}$s identical to their respective control groups. We observed that the ED$_{50}$s in both the "chronic vehicle" and "chronic mismatch" groups in the placebo mice did not differ. Similarly, we observed that threefold tolerance to Δ^9-THC was observed in both the "chronic vehicle" and "chronic mismatch" groups pretreated for 7 days with Δ^9-THC. Thus, the mismatch did not alter tolerance expression. In previous studies (7,22), analgesia induced by the kappa agonist, U50,488H, as well as that of Δ^9-THC, is blocked by antisense oligodeoxynucleotides specific for the kappa$_1$ receptor. In these studies the mismatch groups behaved similarly to the control groups, confirming the specificity of the nucleotide sequence (5′GGTGCCTCCAAGGACTATCGC-3′) for the kappa$_1$ receptor. The timing of the administration of the antisense treatments is also an important factor in studies involving antisense strategies. To observe the downregulation of the kappa$_1$ receptor function, the synthesis of new receptors must be blocked, and sufficient time given to permit the recycling and elimination of preexisting receptors in the membrane. Our protocol for the antisense treatment was identical to Chien et al. (7) and Pugh et al. (22).

Other behavioral effects such as catalepsy and hypoactivity are observed at doses greater than 200 μg/mouse. Motor problems could cause an inhibition of the mouse's ability to flick its tail, although the tail-flick response is a spinal reflex, and is unlikely to be altered by such motor deficits. As the dose was increased, a greater percentage of mice were noted to display splaying of the hindquarters and lethargy. However, Smith et al. (29) demonstrated the separation of the behavioral effects of the cannabinoids from the antinociceptive effects; thus, differing mechanisms or receptors appear responsible for the differing behavioral effects.

Our data clearly demonstrate the expression of tolerance to Δ^9-THC–induced antinociception in the Δ^9-THC/distilled water group. This is consistent with previous reports (11,29). Our data support the previous work (22) that antisense-induced kappa receptor downregulation attenuates the acute antinociceptive effects of the cannabinoids. The degree of downregulation of the kappa receptor by the antisense administration is not known, but has been shown in other studies to decrease by nearly 50% (7). Thus, nearly 50% of kappa receptors presumably remain in the membrane. If downregulation of the kappa$_1$ receptor by antisense administration enhanced Δ^9-THC–induced decreases in kappa receptor number, we would expect the ED$_{50}$ for Δ^9-THC to increase to a greater degree in the antisense/Δ^9-THC–tolerant group than in the antisense/acute Δ^9-THC (nontolerant) group. The ED$_{50}$ for Δ^9-THC in the nontolerant group was increased by 1.9-fold (61 vs. 114 μg/mouse). The ED$_{50}$ for Δ^9-THC in the tolerant group was increased by 2.9-fold (180 vs. 514 μg/mouse). Clearly, some process is activated in animals receiving chronic Δ^9-THC, which enhances the effects observed with antisense/Δ^9-THC. We hypothesize that Δ^9-THC is further downregulating kappa receptor number, an effect that is hypothesized to enhance the effects of the antisense administration. Because the cannabinoids appear to release dynorphin, it is logical to hypothesize that the cellular response to the release of dynorphin might be a downregulation of the kappa receptor to which the dynorphin binds. If such a downregulation of the kappa receptor was enhanced by the elimination of additional kappa receptors via the use of the antisense, then tolerance to Δ^9-THC would be expected to increase. The chronic administration of kappa agonists such as U50,488H have been shown to (1) decrease the kappa receptor affinity through a process of desensitization (3,25); (2) decrease the number of kappa binding sites in brain regions and spinal cord of the rat with no change in affinity (5,36); and (3) decrease kappa receptor affinity in peripheral tissues (44). Conversely, the kappa antagonist nor-BNI has been shown to increase the number of kappa binding sites (5) as well as to increase dynorphin A release in spinal cord (2). Because dynorphin has been shown to be converted to leucine enkephalin, we hypothesize that chronic cannabinoid treatment may also alter delta opioid binding. Chronic administration of delta opioid agonists such as DPDPE has been shown to decrease the number of delta opioid binding sites in rat brain regions with no accompanying changes in receptor affinity (4,32,33), although the mRNA level for the delta receptor has been shown to not be altered following tolerance to the delta agonist deltorphin II (15). Thus, tolerance to the cannabinoids may involve changes in both kappa and delta opioid systems. However, we cannot rule out the possibility of other mechanisms that are also in-

volved in tolerance. Raffa et al. (24) hypothesizes that the G protein subunit, $G_i2\alpha$ might be involved in opioid-induced tolerance expression. This is based on the blockade of morphine-induced antinociception by antisense specific for the $G_i2\alpha$ subunit, and the degree of attenuation produced by different mu agonists. Thus, alterations in G proteins, or an uncoupling at the receptor, could account for cannabinoid-induced tolerance via the interaction of the cannabinoids with kappa-opioids.

In summary, our data support an interaction between Δ^9-THC and the kappa receptor subtype $kappa_1$ in spinally mediated antinociception in mice. The data indicate enhancement of Δ^9-THC–induced tolerance following antisense treatment, which supports the hypothesis that downregulation of the kappa receptor subtype, $kappa_1$, by Δ^9-THC, is responsible at least in part for expression of Δ^9-THC–induced tolerance.

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